

University of Groningen

Improvement of Solubility and Stability of the Antimicrobial Peptide Nisin by Protein Engineering

Rollema, Harry S.; Kuipers, Oscar P.; Both, Paula; Vos, Willem M. de; Siezen, Roland J.

Published in:
International Dairy Journal

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1995

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Rollema, H. S., Kuipers, O. P., Both, P., Vos, W. M. D., & Siezen, R. J. (1995). Improvement of Solubility and Stability of the Antimicrobial Peptide Nisin by Protein Engineering. *International Dairy Journal*, 5(8).

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Improvement of Solubility and Stability of the Antimicrobial Peptide Nisin by Protein Engineering

HARRY S. ROLLEMA,* OSCAR P. KUIPERS, PAULA BOTH, WILLEM M. DE VOS,
AND ROLAND J. SIEZEN

*Department of Biophysical Chemistry, Netherlands Institute for Dairy
Research (NIZO), 6710 BA Ede, The Netherlands*

Received 27 January 1995/Accepted 26 May 1995

Nisin is a 3.4-kDa antimicrobial peptide that, as a result of posttranslational modifications, contains unsaturated amino acids and lanthionine residues. It is applied as a preservative in various food products. The solubility and stability of nisin and nisin mutants have been studied. It is demonstrated that nisin mutants can be produced with improved functional properties. The solubility of nisin A is highest at low pH values and gradually decreases by almost 2 orders of magnitude when the pH of the solution exceeds a value of 7. At low pH, nisin Z exhibits a decreased solubility relative to that of nisin A; at neutral and higher pH values, the solubilities of both variants are comparable. Two mutants of nisin Z, which contain lysyl residues at positions 27 and 31, respectively, instead of Asn-27 and His-31, were produced with the aim of reaching higher solubility at neutral pH. Both mutants were purified to homogeneity, and their structures were confirmed by one- and two-dimensional ^1H nuclear magnetic resonance. Their antimicrobial activities were found to be similar to that of nisin Z, whereas their solubilities at pH 7 increased by factors of 4 and 7, respectively. The chemical stability of nisin A was studied in the pH range of 2 to 8 and at 20, 37, and 75°C. Optimal stability was observed at pH 3.0. Nisin Z showed a behavior similar to that of nisin A. A mutant containing dehydrobutyryne at position 5 instead of dehydroalanine had lower activity but was significantly more resistant to acid-catalyzed chemical degradation than wild-type nisin Z.

Several *Lactococcus lactis* strains are able to produce nisin, an antimicrobial peptide that can inhibit the growth of a wide range of gram-positive bacteria and therefore is used as a preservative in various food products (3, 4). Nisin is a 34-residue-long lantibiotic that contains the unusual amino acid residues dehydrobutyryne (Dhb), dehydroalanine (Dha), lanthionine, and β -methyl lanthionine. The thio-ether bridges of the lanthionines form five ring-like structures in the nisin molecule (Fig. 1). From the primary and tertiary structure of nisin, it can be inferred that the molecule has an amphiphilic character (16, 25). The N-terminal part contains a relatively high number of hydrophobic residues. The C-terminal part is more hydrophilic, containing positively charged side chains of lysine and histidine residues. An additional molecular characteristic of nisin is the absence of residues with negatively charged side chains. This implies that nisin will be positively charged over a wide pH range. At neutral pH, its charge will be reduced by the deprotonation of the histidines, but above pH 7, it will retain a net positive charge because of the high pK values of the lysine residues.

The spatial structure of nisin A in aqueous solution has been determined by nuclear magnetic resonance (NMR) techniques (16, 25). The molecule exhibits a reasonably well-defined structure within the rings formed by the lanthionines. The part of the polypeptide chain connecting rings 3 and 4 and the C-terminal part following ring 5 show an appreciable degree of flexibility.

It has been demonstrated that nisin exhibits the highest solubility and stability at low pH (10). Loss of nisin activity during storage has been reported for several food products (3,

4). However, few systematic studies of nisin A concerning its functional properties such as solubility and chemical stability have been reported (17). A number of degradation products of nisin A have been described (2, 16, 20). In all cases, Dha residues were involved, indicating that the integrity of this unsaturated amino acid is an important factor with respect to the chemical stability of nisin. In particular, modification of Dha at position 5 (Dha5) leads to a severe loss of biological activity. At low pH, the addition of a water molecule to the double bond of Dha can occur, ultimately resulting in the formation of the corresponding amide and keto acid (8). By this mechanism, the polypeptide chain can be cleaved in the first ring at position 5 and/or in the C-terminal part at position 33. In the alkaline pH region, the addition of nucleophiles to the double bond of the unsaturated amino acids can occur, leading to polymerization due to intermolecular reactions (17).

Two natural variants of nisin are known: nisin A (9), the first nisin variant characterized, and nisin Z, which has been reported more recently (18). Nisin A and nisin Z differ by a single amino acid substitution at position 27, being His in nisin A and Asn in nisin Z, and are equally distributed among nisin-producing *L. lactis* strains (5). Recently, expression systems for mutated nisin genes have been developed with *L. lactis* as a host (14, 15). Site-directed mutagenesis of the nisin structural genes allows the construction and production of various mutant nisins to study the relation between structure and biological activity and to alter functional properties such as solubility and chemical stability. This paper presents a detailed study of the solubility and chemical stability of nisin A and nisin Z and describes a number of nisin Z mutants with improved functional properties. The results demonstrate that introduction of lysine residues in nisin considerably enhances its solubility in the neutral pH range and that introduction of a Dhb residue at position 5 results in improved resistance against acid-catalyzed

* Corresponding author. Mailing address: Department of Biophysical Chemistry, Netherlands Institute for Dairy Research (NIZO), P.O. Box 20, 6710 BA Ede, The Netherlands. Phone: 31 8380 59511. Fax: 31 8380 50400.

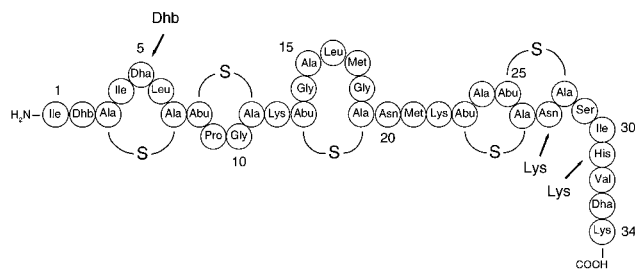


FIG. 1. Schematic representation of the structure of nisin Z. In nisin A, Asn-27 is replaced by His. Arrows indicate the amino acid replacements for the mutants described in this study.

conversion of this unsaturated residue into the corresponding amide and keto acid.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *Escherichia coli* MC1061 (1) was used as a recipient strain in cloning experiments. The *L. lactis* strain used for expression of mutant nisins was NZ9700 (12); it carries the nisin-sucrose transposon Tn5276 (19). Plasmid vectors used for the cloning experiments were pNZ9013 and pNZ9019 (14, 15). *E. coli* strains were grown in TY broth at 37°C. *L. lactis* strains were cultivated without aeration at 30°C in M17 broth (Difco Laboratories, Detroit, Mich.) containing 0.5% (wt/vol) sucrose. For nisin production, cells were grown in SPYS medium (consisting of 1% sucrose, 1% Bacto Peptone [Difco], 1% yeast extract [Difco], 0.2% NaCl, 0.002% MgSO₄ · 7H₂O, and 1% KH₂PO₄ [pH 7.0]) (6). For large-scale production, 3% sucrose instead of 1% sucrose was added. When appropriate, media were supplemented with 10 µg of chloramphenicol per ml for both *E. coli* and *L. lactis*.

DNA manipulations. Plasmid isolations from *E. coli* and transformations of *E. coli* strains were carried out by established procedures (21). Plasmid DNA from lactococcal cells was isolated as described previously (26). DNA was transformed into *L. lactis* by means of electroporation essentially as described previously (26). Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were purchased from New England Biolabs, Inc. (Beverly, Mass.), or Promega Corporation (Madison, Wis.) and used as recommended by the manufacturers. Oligonucleotides used as primers in sequencing and PCRs were synthesized in a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.).

Site-directed mutagenesis of the *nisZ* gene and DNA sequence analysis. Sequences of the primers used for PCR and site-directed mutagenesis are as follows: 5'-GATTAAATTCGACGTTGTAG-3' (*Pst*I), 5'-CCCTAAAAGCTTATAAAATAGG-3' (*Hind*III), 5'-GCTTACGTTACTACATTTACATGTTG-3' (N27K and *Afl*III), 5'-CAGCAACATGTATTGTAGTATTAAGTAAGCAAAT-3' (H31K and *Afl*III), and 5'-GCATTACAAGTATTACACTATGTACACCCGG-3' (S5T). (Sites of nucleotide substitutions are underlined; mutations and restriction sites that were introduced are indicated in parentheses.) Site-directed mutagenesis was performed as described before (13). Sequencing of purified DNA fragments was performed by the dideoxy chain termination method (22).

Production, purification, and characterization of nisin species. Nisin A was purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) either with a nisin-enriched fraction prepared from Nisaplin (nisin A content, 2.5% [wt/wt]; Aplin & Barrett) or with a relatively pure nisin preparation (NBS Biologicals). The nisin-enriched fraction from Nisaplin was prepared by the following procedure. To 20 g of Nisaplin, 250 ml of H₂O was added, the pH was adjusted to 2.0 with HCl, and undissolved material was removed by centrifugation. To the supernatant, NaCl was added to 1.5 M, and the pH was adjusted to 6.3 with NaOH. The precipitated material was centrifuged off and dissolved in 0.05% acetic acid. Wild-type nisin Z and mutants of nisin Z were produced by batch fermentations as described previously (14). Nisin species were purified by chromatography on Fractogel TSK butyl 650-S followed by RP-HPLC as described before (14). All nisin species were checked for purity by analytical RP-HPLC and ¹H NMR. Structures of nisin Z mutants were analyzed by two-dimensional NMR (2D-NMR) techniques essentially as described previously (14).

Solubility studies. A concentrated solution of pure nisin (50 to 5 mg/ml, depending on ionic strength) was prepared in a 0.01 M HCl solution of pH 2.0. The pH was increased gradually by the addition of 0.1 M NaOH solution until the appearance of a precipitate. The sample was equilibrated for 30 min under continuous stirring, and the precipitate was removed by centrifugation (3 min, 15,000 × g). The pH of the supernatant was measured, and the concentration of the saturated nisin solution was determined spectrophotometrically at 220 nm. This procedure was repeated until completion of the solubility versus pH curve (maximum pH, 8.0). All experiments were performed at room temperature (20

± 2°C). The absorptivities of the different nisin species were determined as follows. Pure nisin was dissolved in D₂O (1 to 5 mg/ml). The protein concentration was determined by ¹H NMR with 3-(trimethylsilyl)-propionic 2,2,3,3-⁴-d₄ acid and 2,2,2-trifluoroethanol as reference substances. After appropriate dilution in aqueous 0.05% acetic acid, the A₂₂₀ of the samples was measured with a Cary 1E (Varian) spectrophotometer. The values found for the absorptivity at 220 nm (A₂₂₀) were 18, 16, 16, 14, and 17 ml · mg⁻¹ · cm⁻¹ for nisin A, nisin Z, N27K nisin Z, H31K nisin Z, and Dha5Dhb nisin Z, respectively.

Chemical stability. Nisin solutions (0.05 mg of pure nisin per ml) were prepared at pH 2, 3, 4, 5, 6, 7, and 8. At pH 2 and 3, no buffers were used. At pH 4 and 5, 20 mM sodium acetate was used. In the pH range of 6 to 8, 20 mM sodium phosphate was used. All solutions contained 0.1 M NaCl and 0.02% NaN₃. The samples were incubated at different temperatures. At regular time intervals, samples were taken and the nisin content was determined by RP-HPLC. The incubated samples were also assayed for biological activity against *Micrococcus flavus* DSM1790 at the end of the incubation period or earlier in case a significant decrease in nisin content was expected.

HPLC experiments. RP-HPLC was carried out with a system consisting of a Gilson 231 sample injector, two Waters 510 pumps, a Waters TCM column oven, and a Separations model 759A variable-wavelength detector. Waters Maxima 820 software was used for gradient control and data acquisition. Hi-Pore RP318 (Bio-Rad) columns were used (250 by 4.6 mm for analytical runs [flow rate, 1 ml/min] and 250 by 21.5 mm for preparative runs [flow rate 10 ml/min]). In all experiments, the columns were kept at a constant temperature of 30°C. The elution buffers consisted of 10% aqueous acetonitrile-0.1% trifluoroacetic acid (buffer A), and 90% aqueous acetonitrile-0.08% trifluoroacetic acid (buffer B). A typical gradient used in analytical runs was from 15% to 30% buffer B, linear in 60 min. Peak detection was at 220 nm.

NMR experiments. ¹H NMR spectra were recorded on a Bruker AM400 spectrometer operating at 400.13 MHz. The samples for NMR contained approximately 3 mM nisin in 10% D₂O-90% H₂O (pH 3.5). All spectra were recorded at 25°C. The solvent resonance was suppressed by low-power preirradiation. 3-(Trimethylsilyl)-propionic 2,2,3,3-⁴-d₄ acid was used as a reference for the spectra. Nisin Z mutants were characterized by 2D-NMR techniques as described previously (14).

Biological activity. Antimicrobial activities of (mutant) nisin Z species were determined as MIC values against the indicator strains *M. flavus* DSM 1790, *Streptococcus thermophilus* R₆, and *L. lactis* MG1614 (7) as described before (14) for the first two species. *L. lactis* was grown in M17 medium supplemented with 0.5% glucose at 30°C, with an initial A₆₀₀ of 0.025, and outgrowth was measured when the culture without nisin had reached an A₆₀₀ of 0.8. In the chemical stability studies, the decrease in biological activity was monitored by an agar diffusion assay with *M. flavus* DSM 1790 as the indicator organism (24). In this case, MIC values were determined from dilution series (five concentrations; dilution factor, 1.8) by extrapolation to zero halo. The pH of the medium for the agar plates was adjusted to 7.0. The nisin samples were diluted in 0.05 M phosphate buffer (pH 7.0).

RESULTS AND DISCUSSION

Characterization of nisin mutants. Two nisin Z mutants, N27K nisin Z and H31K nisin Z, were constructed with the aim to improve the solubility at elevated pH values. The mutant Dha5Dhb nisin Z described previously (14) was expected to exhibit enhanced chemical stability. N27K nisin Z and H31K nisin Z were characterized by 2D-NMR techniques to confirm the mutation and the completion of the posttranslational modifications. Both mutant nisins were found to be fully modified and processed. Apart from the mutated residue, the NMR data did not provide any indication of major structural differences as compared with nisin Z. The biological activity of the mutant nisins is shown in Table 1. N27K nisin Z and H31K nisin Z show a biological activity very similar to that of nisin Z, whereas Dha5Dhb nisin Z shows a reduced activity.

Solubility of nisin A. With respect to practical applications, an important functional property of nisin is its solubility. For this reason, it is useful to have data for a wide range of experimental conditions. It has been reported that the solubility of nisin depends strongly on the pH and buffer concentration used (10, 17), but there are no systematic studies covering a broad range of experimental conditions. We have determined the solubility of nisin A in a wide pH range (i.e., 2 to 8) and ionic strength range (0 to 1.5 M NaCl). Figure 2 shows the pH dependence of the solubility of nisin A at various ionic strengths. At low pH and low ionic strength (approximately

TABLE 1. Antimicrobial activities of nisin A, nisin Z, Dha5Dhb nisin Z, N27K nisin Z, and H31K nisin Z against *M. flavus* DSM 1790, *S. thermophilus* R₈, and *L. lactis* MG1614

Nisin	MIC ($\mu\text{g/liter}$) ^a		
	<i>M. flavus</i>	<i>S. thermophilus</i>	<i>L. lactis</i>
Nisin A	11	6	15
Nisin Z	11	6	15
Dha5Dhb nisin Z	21	65	140
N27K nisin Z	10	6	17
H31K nisin Z	16	10	34

^a Standard errors were less than 15% of each given value.

0.01 M NaCl), the solubility exceeds 40 mg/ml. In the pH range studied, the solubility continuously decreases with increasing pH and shows a greater-than-10-fold reduction when the pH is increased from 3.0 to 7.0. At all pH values, a strong effect of ionic strength is observed. At NaCl concentrations above 1 M, the pH dependence nearly vanishes and the solubility drops far below 1 mg/ml in the whole pH range. The values found for the solubility in our study seem to be lower than those cited in some reviews (10, 11), but there is a reasonable agreement between them and the data of Liu and Hansen (17). The strong influence of ionic strength on the solubility demonstrates that electrostatic interactions play an important role with respect to the stabilization of nisin in solution. The pH dependence of the solubility does not clearly reflect the pK values of the titratable residues, i.e., the C-terminal carboxyl with a pK of 3 to 4 and the histidine residues with a pK of 6.5 (23). Apparently, the pH dependence of the solubility of nisin A is not governed solely by the titratable amino acid residues.

The present results show that in the acid and neutral pH range at moderate ionic strength, the solubility of nisin A is at least 3 to 4 orders of magnitude higher than its MIC values towards different target organisms. However, at high ionic strength and neutral pH (and above), the solubility could approach the dose required for some applications, such as the brining of cheeses. In these cases, the solubility could well be a limiting factor with respect to practical applications.

Solubility of nisin A, nisin Z, N27K nisin Z, and H31K nisin Z. The solubilities of nisin A, nisin Z, N27K nisin Z, and H31K nisin Z as a function of pH at 0.15 M NaCl were compared (Fig. 3). The two mutant nisin Z species could be studied only in a relatively narrow pH range because of a limitation in the amount of material available. Below pH 5.0, nisin Z has a lower solubility than nisin A because of the His to Asn muta-

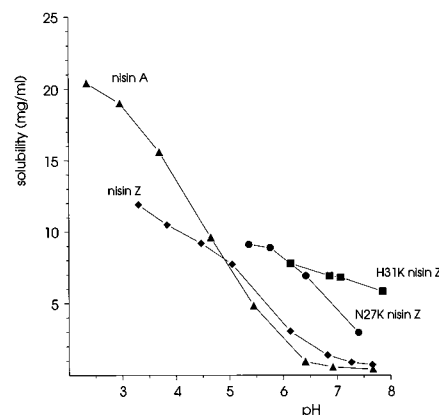


FIG. 3. Solubility of nisin A, nisin Z, N27K nisin Z, and H31K nisin Z as a function of pH at 20°C and 0.15 M NaCl.

tion at position 27 which reduces the charge of the molecule at low pH. Above pH 5.0, the solubility of nisin Z is slightly higher than that of nisin A, probably because of the more-polar character of the Asn residue compared with that of a deprotonated His residue. In the neutral pH region, the two nisin Z mutants containing an additional lysine residue show a significantly increased solubility as compared with that of nisin Z. In N27K nisin Z, an additional positive charge is introduced. In the case of H31K nisin Z, the pK of the titratable group at position 31 is increased considerably. Both mutations have a marked effect on the solubility. At pH 7, a fourfold-higher solubility is observed for N27K nisin Z than for nisin Z. H31K nisin Z is even more soluble than N27K nisin Z, because its solubility increases by a factor of seven relative to that of nisin Z.

Chemical stability of nisin A. The use of nisin as a preservative in foods poses specific demands with respect to chemical stability. A primary condition is that nisin should survive heat treatments applied in food processing. Second, a reasonably long-term stability is required in the case of application in products with a long shelf-life. It is known that nisin is not stable at high pH (10, 17). The main reason for this instability is the reactivity of the unsaturated amino acids which can undergo a variety of addition reactions at alkaline pH (8). It has been reported that nisin, when applied in certain food products, shows a slow loss of activity during storage (3, 4), indicating that in the neutral-to-acid pH region as well, the long-term stability of nisin is limited. We have studied the chemical stability of nisin A at 20, 37, and 75°C in the pH range of 2 to 8. Nisin solutions were incubated at different pH values, and the RP-HPLC patterns were monitored as a function of time. After a major decrease in nisin content, as judged from HPLC or at the end of the incubation period, the biological activity towards *M. flavus* was determined in an agar diffusion assay. Figure 4 shows the decrease in nisin A content as determined from HPLC as a function of time and pH at 20 and 37°C. In Fig. 5, the stabilities of nisin A and nisin Z at 75°C are compared. In all experiments, a reasonable correlation between the decrease in nisin content derived from HPLC and the decrease in biological activity towards *M. flavus* was observed. A typical example is shown in Fig. 6. The consistently higher biological activity relative to the amount of intact nisin as measured by RP-HPLC could be explained by the fact that some degradation products, e.g., nisin A(1-32), retain full or slightly reduced biological activity (2, 20).

At room temperature, nisin A is relatively stable in the pH range of 2 to 6. In this pH range, only a moderate effect of pH

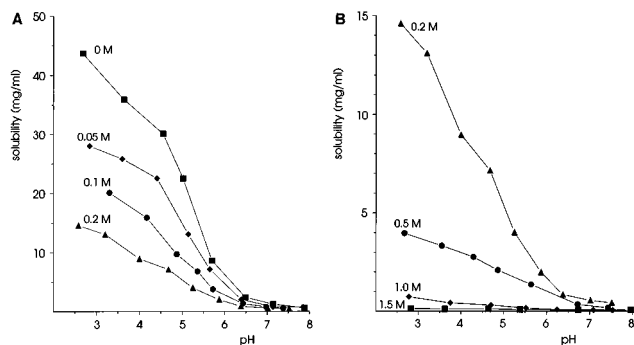


FIG. 2. Solubility of nisin A at 20°C as a function of pH at different NaCl concentrations (0 to 0.2 [A] and 0.2 to 1.5 [B]). Note the difference in the y-axis scale between panels A and B.

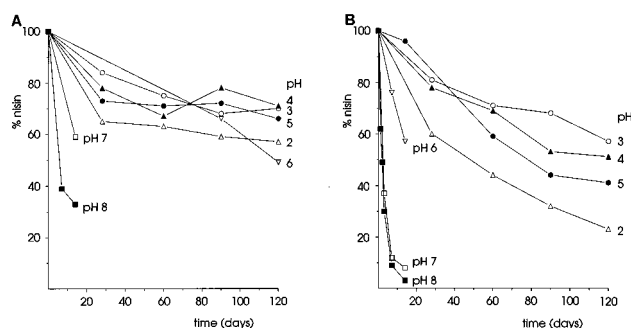


FIG. 4. Chemical stability of nisin A at 20°C (A) and 37°C (B). Residual nisin was determined by RP-HPLC as a function of incubation time at different pH values (values indicated in the figure). Conditions were 0.05 mg of nisin per ml, 0.1 M NaCl, and 0.02% NaN_3 .

on the chemical stability is observed. A sharp decrease in stability is observed at pH 7 and 8. At 37 and 75°C, a more distinct pH dependence is observed. The highest stability is observed at pH 3.0. At pH values both lower and higher than 3.0, the chemical stability is reduced. In the neutral pH region and at an elevated pH, a much lower stability is observed than that at low pH values. At 75°C, nisin Z shows a chemical stability very similar to that of nisin A. The results indicate that nisin has, especially at low pH, a good short-term stability, i.e., sufficient to survive most heat treatments applied in food processing. On the other hand, it becomes clear that the long-term chemical stability of nisin, particularly at elevated temperatures, is limited. The latter observation could be of significance with respect to application of nisin in food products with a long shelf-life. Little is known about the mechanism of nisin degradation at moderate pH values. The HPLC results show complex patterns, which are pH dependent. This indicates that the degradation mechanism is complex and that many degradation products can be formed. It should be realized that the data presented here apply to nisin in an aqueous buffered solution, giving information on the intrinsic stability of nisin. When nisin is applied in different food products, its stability could be influenced by the matrix of the product as such and/or by components which interact with nisin.

Chemical stability of Dha5Dhb nisin Z. Since the integrity of the relatively labile Dha5 is essential for the biological activity of nisin and since all nisin degradation products characterized to date contain an intact Dhb residue at position 2 (2,

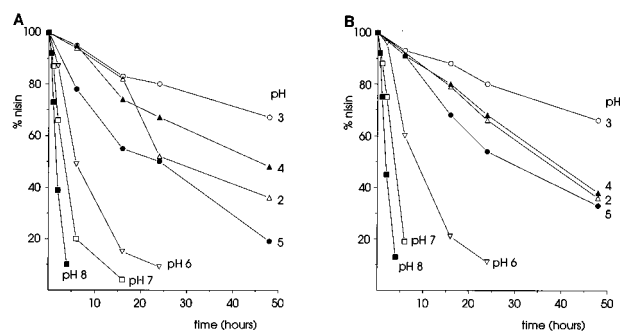


FIG. 5. Chemical stability of nisin A (A) and nisin Z (B) at 75°C. Note the difference between the time scale in this figure and the one in Fig. 4. Residual nisin was determined by RP-HPLC as a function of incubation time at different pH values (values are indicated in the figure). Conditions were 0.05 mg of nisin per ml, 0.1 M NaCl, and 0.02% NaN_3 .

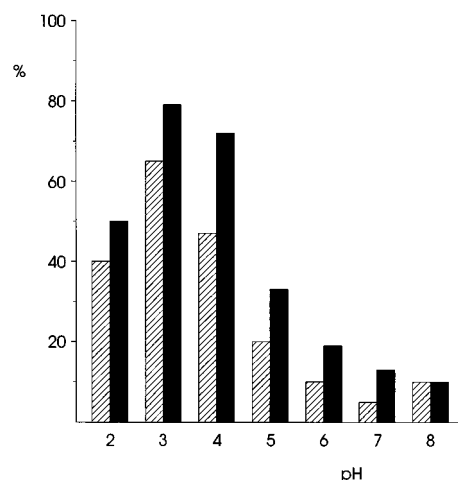


FIG. 6. Comparison of residual nisin A content as determined by RP-HPLC (shaded bars) and residual biological activity (filled bars) at different pH values during incubation at 75°C. Conditions were as described in the legend to Fig. 5. Incubation times: 48 h at pH 2 to 5, 24 h at pH 6, 6 h at pH 7, and 4 h at pH 8.

20), a mutant of nisin Z in which Dha5 was replaced by a Dhb residue was designed. This mutant was expected to be more stable than nisin Z.

From studies of nisin degradation products, it is known that Dha residues can undergo an acid-catalyzed addition of a water molecule to the double bond, resulting in the formation of an α -OH-Ala residue (8, 20). This product is not stable and is converted to the corresponding amide and keto acid. This conversion can be realized in a controlled way by freeze-drying with 0.1 M HCl. Figure 7 shows the effect of repeated freeze-drying on the RP-HPLC pattern of Dha5Dhb nisin Z and nisin Z. In the case of the mutant, the number of degradation prod-

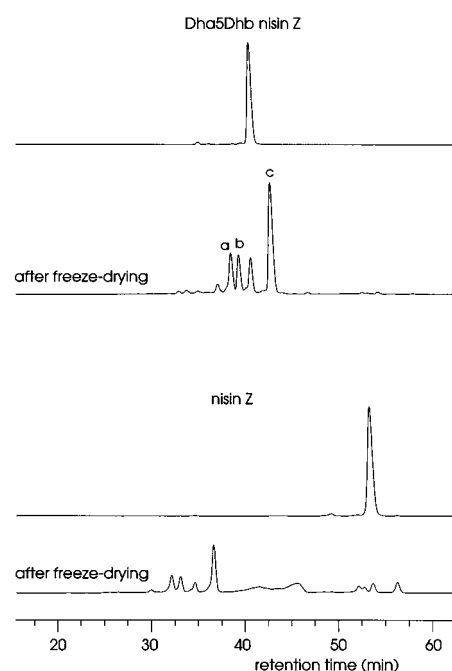


FIG. 7. Effect of freeze-drying repeated three times with a 0.1 N HCl solution on the RP-HPLC pattern of Dha5Dhb nisin Z (upper traces) and nisin Z (lower traces).

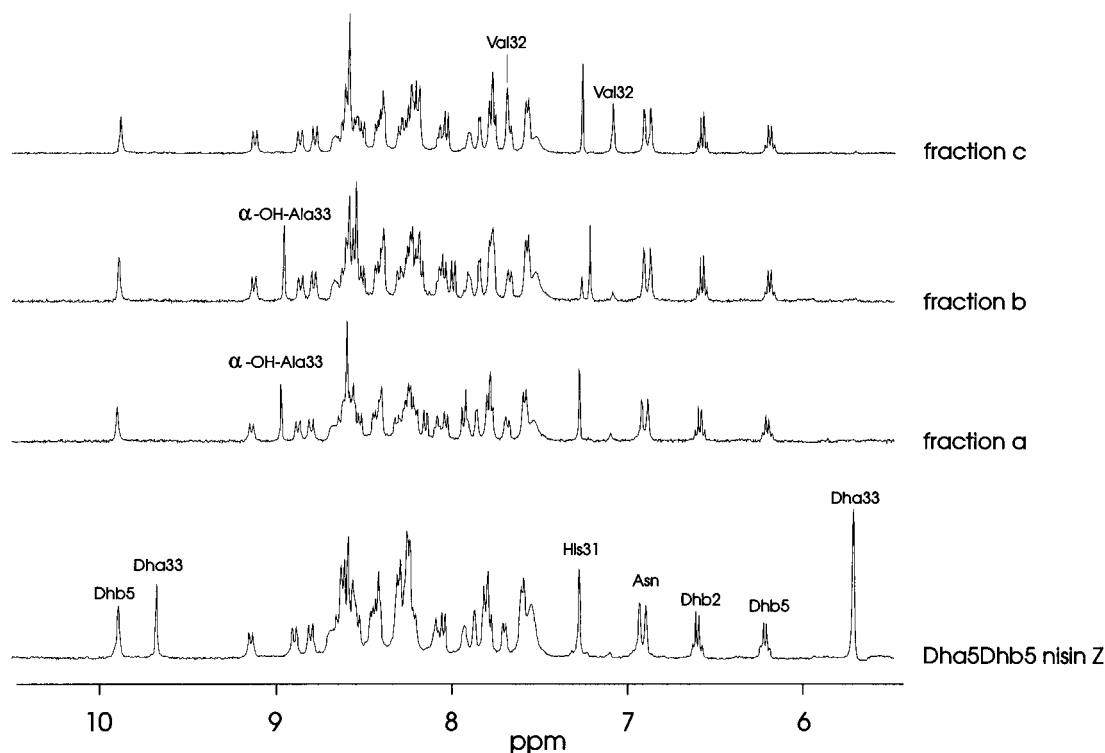


FIG. 8. Low-field part of ^1H NMR spectrum of the different components of Dha5Dhb nisin Z shown in Fig. 7. The assignment of a number of resonances is indicated in the figure. Conditions were 90% H_2O –10% D_2O , pH 3.5, and 25°C .

ucts formed is limited. In the case of nisin Z, a more-extensive degradation is observed. The changes in the RP-HPLC pattern of Dha5Dhb nisin Z as a function of the number of freeze-drying cycles showed that first two intermediate species with a retention time slightly shorter than that of the parent species are formed (Fig. 7, peaks a and b). Subsequently, these components are both converted to a stable component with a longer retention time than that of the starting material (Fig. 7, peak c). Components a, b, and c were isolated by preparative RP-HPLC and characterized by 2D-NMR. Components a and b appeared to be $[\alpha\text{-OH-Ala-33, Dhb5}]$ nisin Z. Component c was identified as Dha5Dhb nisin Z(1-32). Figure 8 shows the low-field part of the ^1H NMR spectra of the different components. Apart from the backbone amide resonances, the spectrum of Dha5Dhb nisin Z shows a number of characteristic resonances from the unsaturated amino acids, from His-31, and from the two Asn residues. The spectra of components a and b lack the vinyl resonance of Dha33 at 5.73 ppm, and the resonance of the amide proton of $\alpha\text{-OH-Ala-33}$ is seen as a singlet at 8.99 ppm. In the spectrum of component c, the latter resonance has disappeared and two new resonances of the C-terminal amide of Val-32 are observed at 7.10 and 7.71 ppm. The structural difference between components a and b could not be resolved. The 2D-NMR spectra were very similar; only differences in chemical shift for the residues close to position 33 were observed, indicating that these two components have similar primary structures. A possible interpretation could be that these two components represent the two stereochemical isomers of the $\alpha\text{-OH-Ala}$ residue at position 33. From Fig. 7, it can be seen that freeze-drying repeated three times results in a considerable degradation of nisin Z, whereas Dha5Dhb nisin Z is converted to Dha5Dhb nisin Z(1-32) by degradation only at Dha33 near the C terminus. In the case of nisin Z, a nearly

complete loss of activity was observed. In the case of Dha5Dhb nisin Z, the decrease in activity was negligible. This is in accordance with observations that degradation of Dha33 does not lead to loss of biological activity (2, 20). Figure 9 shows a comparison of the chemical stabilities of nisin Z and Dha5Dhb nisin Z(1-32) by measuring the remaining antimicrobial activity at 75°C and different pH values. At pH 1, Dha5Dhb nisin Z(1-32) is considerably more stable than nisin Z. In the pH range of 2 to 6, however, both species show similar stabilities. The large difference in stability observed in the case of freeze-

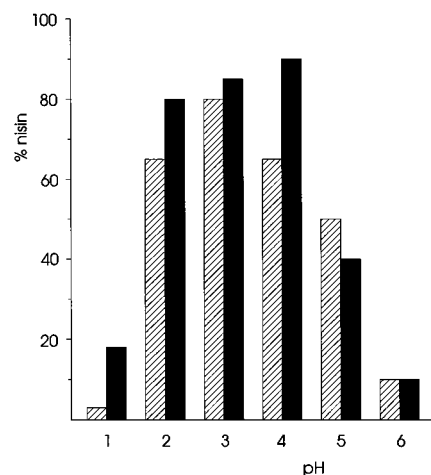


FIG. 9. Comparison of the levels of pH dependence of the chemical stability of nisin Z (shaded bars) and Dha5Dhb nisin Z(1-32) (filled bars) at 75°C . Conditions were as described in the legend to Fig. 5; incubation time was 24 h.

drying, which is a reflection of stability at a pH of <2, is not observed in the pH range of 2 to 6. This indicates that Dha5 plays a major role in the inactivation of nisin at low pH but also that other processes can contribute significantly to the degradation process.

Conclusions. The results of the present study provide information on the solubility and chemical stability of nisins A and Z in a broad range of experimental conditions. The solubility of nisin is strongly dependent on pH and ionic strength. The highest solubility is observed at low pH and low ionic strength. The chemical stability of nisin at room temperature shows little pH dependence in the pH range of 2 to 6. At 37 and 75°C, optimal stability is observed at pH 3. Solubility and stability of nisin Z can be significantly improved by protein engineering, while the antimicrobial activity and spectrum remain similar. Introduction of lysine residues in nisin Z improves the solubility at neutral pH. Replacement of Dha5 by Dhb improves the resistance to acid-catalyzed addition of water to the double bond of this residue. In the present paper, the enhancement of solubility and chemical stability is demonstrated in different mutants. Combination of mutations in one variant could very well lead to a species with both improved solubility and stability.

ACKNOWLEDGMENTS

We are grateful to Hein Boot for construction of mutant nisin genes, Lilian Vreede for determining MIC values against *L. lactis*, and Charles Slangen for assistance with the HPLC experiments.

This work was supported by contract BIOT-CT91-0265 of the BRIDGE program on Lantibiotics of the Commission of the European Communities.

REFERENCES

- Casadaban, M. J., and S. N. Cohen. 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. *J. Mol. Biol.* **143**:179–207.
- Chan, W. C., B. W. Bycroft, L.-Y. Lian, and G. C. K. Roberts. 1989. Isolation and characterization of two degradation products derived from the peptide antibiotic nisin. *FEBS Lett.* **252**:29–36.
- Delves-Broughton, J. 1990. Nisin and its uses as a food preservative. *Food Technol.* **44**:100–117.
- Delves-Broughton, J. 1990. Nisin and its application as a food preservative. *J. Soc. Dairy Technol.* **43**:73–76.
- De Vos, W. M., J. W. M. Mulders, R. J. Siezen, J. Hugenholtz, and O. P. Kuipers. 1993. Properties of nisin Z and the distribution of its gene, *nisZ*, in *Lactococcus lactis*. *Appl. Environ. Microbiol.* **59**:213–218.
- De Vuyst, L., and E. J. Vandamme. 1992. Influence of the carbon source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentations. *J. Gen. Microbiol.* **138**:571–578.
- Gasson, M. J. 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* **154**:1–9.
- Gross, E. 1977. α,β -Unsaturated and related amino acids in peptides and proteins, p. 131–153. In M. Friedman (ed.), *Protein crosslinking. Nutritional and medical consequences*. Plenum Press, New York.
- Gross, E., and J. L. Morell. 1971. The structure of nisin. *J. Am. Chem. Soc.* **93**:4634–4635.
- Hurst, A. 1981. Nisin. *Adv. Appl. Microbiol.* **27**:85–123.
- Hurst, E., and D. G. Hoover. 1993. Nisin, p. 369–394. In P. M. Davidson and A. L. Branen (ed.), *Antimicrobials in foods*. Marcel Dekker, Inc., New York.
- Kuipers, O. P., M. M. Beerthuyzen, R. J. Siezen, and W. M. de Vos. 1993. Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*. *Eur. J. Biochem.* **216**:281–291.
- Kuipers, O. P., H. J. Boot, and W. M. de Vos. 1991. Improved site-directed mutagenesis method using PCR. *Nucleic Acids Res.* **19**:4558.
- Kuipers, O. P., H. S. Rollema, W. M. G. J. Yap, H. J. Boot, R. J. Siezen, and W. M. de Vos. 1992. Engineering dehydrated amino acid residues in the antimicrobial peptide nisin. *J. Biol. Chem.* **267**:24340–24346.
- Kuipers, O. P., W. M. G. J. Yap, H. S. Rollema, M. M. Beerthuyzen, R. J. Siezen, and W. M. de Vos. 1991. Expression of wild-type and mutant nisin genes in *Lactococcus lactis*, p. 250–259. In G. Jung and H.-G. Sahl (ed.), *Nisin and novel lantibiotics*. ESCOM, Leiden, The Netherlands.
- Lian, L.-Y., W. C. Chan, S. D. Morley, G. C. K. Roberts, B. W. Bycroft, and D. Jackson. 1992. Solution structures of nisin A and its two major degradation products determined by n.m.r. *Biochem. J.* **283**:413–420.
- Liu, W., and J. N. Hansen. 1990. Some chemical and physical properties of nisin, a small-protein antibiotic produced by *Lactococcus lactis*. *Appl. Environ. Microbiol.* **56**:2551–2558.
- Mulders, J. W. M., I. J. Boerrigter, H. S. Rollema, R. J. Siezen, and W. M. de Vos. 1991. Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. *Eur. J. Biochem.* **201**:581–584.
- Rauch, P. J. G., and W. M. de Vos. 1992. Characterization of the novel nisin-sucrose conjugative transposon Tn5276 and its insertion in *Lactococcus lactis*. *J. Bacteriol.* **174**:1280–1287.
- Rollema, H. S., P. Both, and R. J. Siezen. 1991. NMR and activity studies of nisin degradation products, p. 123–130. In G. Jung and H.-G. Sahl (ed.), *Nisin and novel lantibiotics*. ESCOM, Leiden, The Netherlands.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Sanger, F. S., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
- Slijper, M., C. W. Hilbers, R. N. H. Konings, and F. J. M. van de Ven. 1989. NMR studies of lantibiotics. Assignment of the ^1H -NMR spectrum of nisin and identification of interresidual contacts. *FEBS Lett.* **252**:22–28.
- Tramer, J., and G. G. Fowler. 1964. Estimation of nisin in foods. *J. Sci. Food Agric.* **15**:522–528.
- Van de Ven, F. J. M., H. W. van den Hooven, R. N. H. Konings, and C. W. Hilbers. 1991. NMR studies of lantibiotics. The structure of nisin in aqueous solution. *Eur. J. Biochem.* **202**:1181–1188.
- Vos, P., G. Simons, R. J. Siezen, and W. M. de Vos. 1989. Primary structure and organization of the gene for a procaryotic, cell-envelope located serine proteinase. *J. Biol. Chem.* **264**:13579–13585.